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Sampling and analysis of volatile organic compounds in bovine breath by solid-phase microextraction and gas chromatography–mass spectrometry

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Abstract

A relatively noninvasive method consisting of a face mask sampling device, solid-phase microextraction (SPME) fibers, and a gas chromatography–mass spectrometry (GC–MS) for the identification of volatile organic compounds (VOCs) in bovine breath was developed. Breath of three morbid steers with respiratory tract infections and three healthy steers were sampled seven times in 19 days for 15 min at each sampling. The breath VOCs adsorbed on the divinylbenzene (DVB)–Carboxen–polydimethyl siloxane (PDMS) 50/30 μm SPME fibers were transported to a laboratory GC–MS system for separation and identification with an in-house spectral library of standard chemicals. A total of 21 VOCs were detected, many of them for the first time in cattle breath. Statistical analyses using Chi-square test on the frequency of detection of each VOC in each group was performed. The presence of acetaldehyde ($P \leq 0.05$) and decanal ($P \leq 0.10$) were associated more with clinically morbid steers while methyl acetate, heptane, octanal, 2,3-butadione, hexanoic acid, and phenol were associated with healthy steers at $P \leq 0.10$. The results suggest that noninvasive heath screening using breath analyses could become a useful diagnostic tool for animals and humans.

Keywords: Air analysis; Volatile organic compounds

1. Introduction

Bovine respiratory tract diseases are prevalent in the beef cattle industry and account for 80% of deaths in the feedyard. The annual economic losses have been estimated at US$ 800–900 million in the USA. [1]. Techniques for early intervention include nutrition, antibiotic therapy, and frequent observation. Physicians, veterinarians, and research scientists are seeking noninvasive methods for the diagnosis, monitoring, and study of disease states. Noninvasive methods reduce the stress and discomfort in animals and humans with little alteration in the physiology of the subject. To date, there are no noninvasive techniques for using unique breath biomarkers that could indicate stress or disease status of beef cattle. Animal and human breath sampling has been used for the identification of metabolic end products such as hydrogen, methane, volatile fatty acids (VFAs), and other volatile organic compounds (VOCs). The tritium (^{3}H) breath test is used to detect malabsorption of foods in dietary tests [2–4]. Large animals such as horses have been monitored with the tritium breath test [5]. The urea (^{13}C) breath test has been used to detect the presence of bacterial infection in humans [6] and in cats and monkeys [7,8]. The ^{3}H and the ^{13}C breath tests are isotopic tracer techniques and require special sample preparation, handling and analysis. The cost of using isotope labeling could be very prohibitive especially under production conditions.

Breath sampling can be used to measure the level of oxidative stress in humans [9]. Aghdassi and Allard [10] reported the relationship between breath alkanes and oxidative stress in human subjects. To date, about 3000 compounds have been detected in human breath at least once using sorbent tubes, yet only 27 of those compounds were found in every human sampled ($n = 50$) suggesting that many compounds are unique to the individual sampled [11]. Acetone, acetaldehyde, ammonia, ethanol and water have been measured in real time in humans with selected ion flow tube mass spectrometry (SIFT-MS) [12]. Lindinger et al. [13] have also used proton-transfer-reaction mass spectrometry (PTR-MS) to detect acetone, various disulfides, isoprene, methanol, acetonitrile, and benzene in human breath.

Solid-phase microextraction (SPME) has been used for sampling of human breath [14,15]. Grote and Pawliszyn [14] quantified isoprene, ethanol, and acetone in human breath.

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A method using membrane extraction with sorbent interface (MESI) has been used to monitor acetone, isoprene, ethanol, and methanol levels in humans [16]. Spinhirne et al. [17] showed the feasibility of SPME and GC–MS for sampling and analysis of cattle breath VOCs. Cattle breath has been sampled with sorbents, sensor array (i.e. electronic nose), and Fourier transform infrared spectroscopy (FT-IR) for the measurement of acetone and other VOCs for indication of health of underfed dairy cows [18,19]. To date, there are a few reported bovine breath VOC studies which attest to the difficulty of breath sampling, concentration, and analysis. Although cattle breath may differ somewhat from human breath, it is conceivable that there are some similarities due to systemic metabolism of both humans and cattle.

This research focused on using a modified sampling system from previous work with bovine breath sampling [17]. The device for on-site, noninvasive animal breath sample collection was designed, built, and tested. Breath sampling was achieved with SPME, which combined sampling and sample preconcentration, facilitated sample preservation, and used to inject the sample into a GC for VOC speciation. The objective of this research was to identify VOCs in the breath of both healthy and sick steers and to identify unique biomarkers of respiratory disease present in the breath of the sick animals. This noninvasive breath sampling method could become a convenient way to monitor and study disease states in cattle and humans.

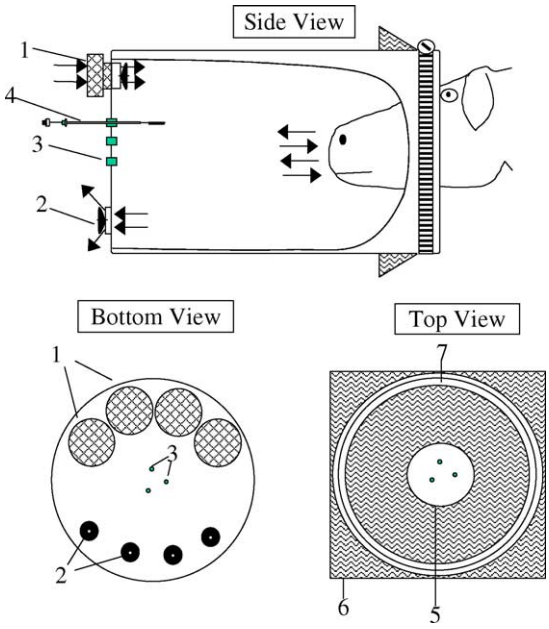

Fig. 1. Device for sampling of VOCs in bovine breath with SPME. The air entering the sampling chamber is filtered (1), and the exiting air is kept separate with check valves (2). Septa (3) allow sampling with SPME (4). The animal's nose is placed the hole (5) in the flexible silicone sheet (6) that is held in place with a large diameter clamp (7).

2. Experimental

2.1. Breath sampling device

Although similar to the Spinhirne et al. [17] prototype, the modified sampling system used to collect bovine breath was improved in several ways to decrease background by 68% and consisted of a modified 28.3 l, stainless steel bucket equipped with a 1.5 cm thick silicone sheet (Diversified Silicone, Santa Fe Springs, CA, USA) in place of the lid. Two 18 cm hose clamps were connected end to end to form a band that secured the silicone sheet to the top of the bucket. One-way valves and filters for the North 770-30M industrial safety respirator (North Safety Products, Cranston, RI, USA) were installed at the base of the face mask for removal of background gases including VOCs, hydrogen sulfide, ammonia, and methylamines from air entering the sampling device. The number of filters and outlet valves was increased to four to assure that the animal was receiving adequate breathing air supply and to reduce leaks around the sealing membrane due to hyperventilation. The filters (inlets) were placed towards the top of the container and the outlet valves were placed near the bottom of the sampling system in a manner that allow mucus from the animal to drain from the system without damaging the filters during samplings (Fig. 1). The same type of filter cartridges, septa, and outlet valves were used in the stainless steel buckets as were used with the high-density polyethylene (HDPE) system [17]. Only the overall diameter of the silicone sheet, the number and placement of filter cartridges and outlet valves, and the internal volume of the container were increased.

All parts of the sampling system except for the filters were cleaned with laboratory detergent and warm tap water followed by rinsing with demonized water. Additionally, the stainless steel containers were treated once with a 10% nitric acid solution for 5 min to passivate the stainless steel surface before initial assembly. This was followed by rinsing with demonized water. Also, the steel construction allowed heating in the drying oven at 210 °C to dry off water and purge other contaminants from the surface of the sampling system. Sampling system blanks were taken with SPME for 15 min inside a sealed system before the hole in the silicone sheet was cut. The stainless steel added very little to the background of the SPME samples and was a preferable material to HDPE. Six sampling devices were assembled and assigned to an individual animal.

After each sampling event, the sampling devices were totally disassembled. All the parts except the filter cartridges were washed with laboratory detergent and warm water. The check valves were air dried and not baked because of obvious heat damage that would result in the drying oven. The silicone sheets were heated at 150 °C for 1 h. The stainless steel containers were also heated at 210 °C for 1 h, allowed to cool, and then assembled with the same filters, cleaned check valves and cleaned silicone sheets used in the previous sampling. New septa were installed in the sampling

devices to avoid any carryover from previous samplings because they were less expensive.

After each cleaning of the sampling devices, in-lab sampling devices blanks were taken to characterize their VOC background. To obtain the blanks, the sampling devices were sealed with an extra silicone sheet and then inverted on the laboratory bench top. With the sealing silicone sheet acting as a diaphragm, air was manually pumped through the sampling device using the attached filters and check valve system. A single divinylbenzene (DVB)–Carboxen–polydimethyl siloxane (PDMS) 50/30 μm SPME fiber was dedicated to sampling background from the sampling devices. One blank was collected from each sealed sampling device in a 15 min extraction. Sampling system blanks were used to evaluate the cleaning protocol and identify contamination stemming from the sampling device.

2.2. Sampling plan

A group of 10 crossbred beef steers were selected from a larger shipment of cattle. The 10 animals were classified as sick ($n = 5$) and healthy ($n = 5$) with a visual scoring system [1]. Of those animals, three healthy and three sick animals were randomly selected for the study, and were sampled seven times in 19 days for 15 min at each sampling. All other cattle were used as replacements. The animals were positioned in a hydraulic squeeze chute that controlled their movements and allowed access to their head and face for placement of the sampling device. The animals were allowed to enter the chute in random order. However, special care was taken to match each sampling device to its assigned steer. One steer died from his advanced respiratory illness after the first sampling, and was replaced by another sick steer.

2.3. SPME sampling

To prepare for sampling with the stainless steel buckets, 23 DVB–Carboxen–PDMS 50/30 μm conditioned SPME fibers were desorbed for 10 min in the GC injector and packaged in preservation vials [17]. The DVB–Carboxen–PDMS 50/30 μm fibers are commercially available from Supelco and are comprised of one solid layer each of DVB (50 μm) and Carboxen (30 μm) held onto the fused silica core by a layer of PDMS. The solid layers are the outermost and impart the fiber with their adsorptive characteristics. At each sampling, triplicate DVB–Carboxen–PDMS 50/30 μm fibers were used to collect samples from each animal simultaneously. The DVB–Carboxen–PDMS 50/30 μm fiber coating was selected because we targeted VOCs and use relatively short sampling times. [17]. Each bucket/steer/fibers combination was maintained throughout the seven samplings to minimize variations in fiber coatings, and sampling systems. The fibers were transported with two trip blanks and three air blanks to the laboratory for analysis with GC–MS.

Sampling took place on seven different occasions (days 1, 3, 5, 11, 15, 17, and 19) at the Texas Agricultural Experiment Station Research Feedlot at Bushland, TX, USA. The sampling device was first inserted over the nostril/jaw area (Fig. 1) purged with the breath of the animal for 3 min before sampling with SPME. Triplicate SPME samples were taken simultaneously to collect the breath VOCs from each steer. Trip blanks were kept in their preservation vials, taken to the sampling site, and were analyzed to test the background from the preservation apparatus. Ambient air samples were also collected with the same type of SPME fiber coating for 15 min at the same time as the breath samples to determine if contaminants from outside the bucket interfered with breath samples. Each sample was capped with a PTFE plug, placed inside a glass culture vial, and placed on ice for transportation to the laboratory GC–MS to preserve the adsorbed breath components on the SPME coating [17,20]. The mucus from the animals was expected to contain some VOCs that could partition to the headspace [21] of the sampling device. The breath of the animal was exposed to the mucus inside the animal as well. However, the measurement of the VOCs within mucus was not in the scope of this research.

2.4. Gas chromatography–mass spectrometry (GC–MS) methods

The model 3800 GC–MS (Varian, Walnut Creek, CA, USA) method was described in Spinhirne et al. [17] except the analysis was completed on a 30 m × 0.25 mm, 0.25 μm film ZB-Wax capillary column (Phenomenex, Torrance, CA, USA). A new Saturn user library was created with the new column to identify compounds in breath and blank samples. A list of the GC–MS method parameters are listed in Table 1.

Table 1
SPME and analysis conditions

Fiber	DVB–Carboxen–PDMS 50/30 μm
Extraction	15 min at ~30 °C (T measured once only)
Desorption	7 min at 250 °C
GC conditions	
Column	ZB-Wax 30 m × 0.25 mm, 0.25 μm
Oven	60–110 °C at 60 °C/min to 210 °C at 10 °C/min to 250 °C at 60 °C/min (6 min hold)
Carrier gas	1 ml/min (constant flow)
Injection port	Splitless, 0.8 mm liner, graphite ferrule, 250 °C
MS conditions	
Mass window	35–200 (m/z)
Transfer line	200 °C
Manifold	40 °C
Trap	150 °C
Current	10 μm
Scans/s	2.27
Multiplier	1340 eV

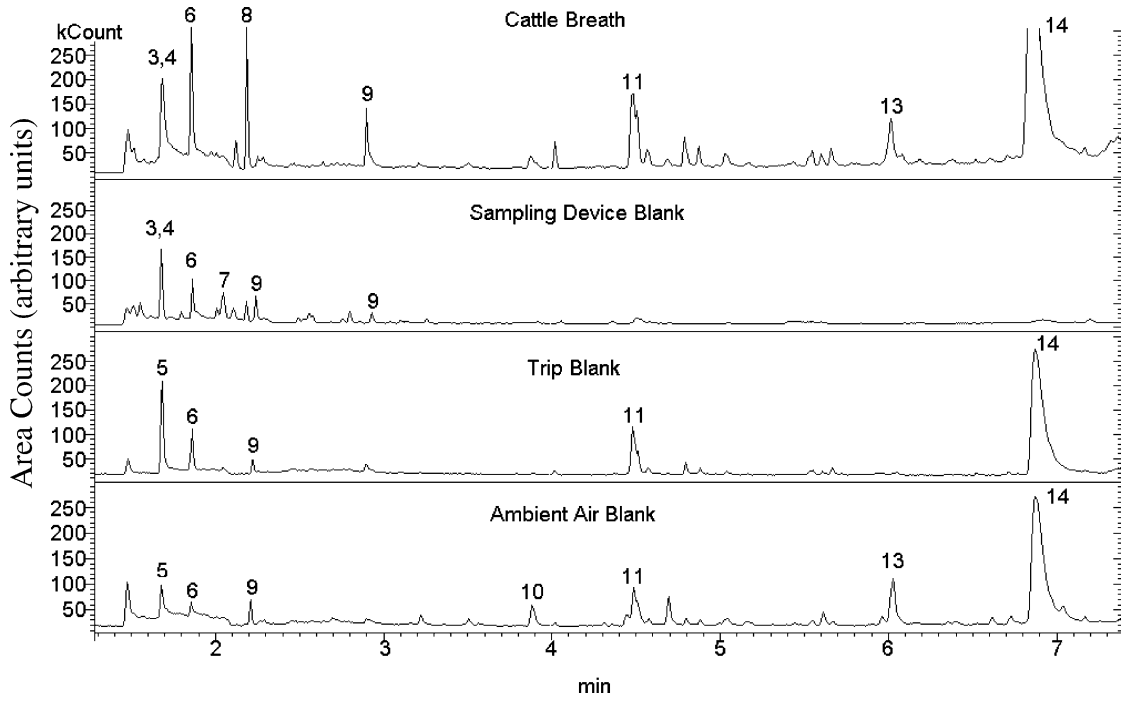

Fig. 2. Chromatogram of cattle breath compared to the chromatograms of the sampling system blank (before going to the field), a trip blank (never taken out of preservation vial), and an air blank (air outside the container at the same time breath is sampled). Peak numbers correspond to those found in Table 2.

Table 2
A list of VOCs collected with SPME from cattle breath and identified by GC/MS

No.	Compound	Retention time (min)	Area	Fit	Reverse fit	Purity	CAS No.
Identified peaks							
1	Hexane	1.522	10113[a]	848	804	794	110-54-3
2	Heptane	1.516	13650[a]	796	784	734	142-82-5
3	Acetone	1.696	110475[a]	919	378	348	67-64-1
4	Acetaldehyde	1.696	90309[a]	734	232	171	75-07-0
8	Toluene	2.185	26030[a]	837	280	254	108-88-3
12	Isovaleric acid	5.954	13670[a]	872	522	497	503-74-2
Unidentified peaks							
5	3,3′-(1,2-Ethenediyl)bis-Thiophene	1.685	262467[b]	456			
6	Polyaromatic hydrocarbons	1.859	302444[b]	654			
7	Trimethylsilanol	2.044	105722[b]	657			
9	Tetramethyl silane	2.897	28801[b]	518			
10	Nonanal	3.885	73487[b]	687			
11	4,6-Dimethyoxy-2,3-dimethyl-benzaldehyde	4.476	22730[b]	564			
13	4-Hydroxybutyric acid	6.013	185298[b]	501			
14	2-(Trimethylsilyl) phenol	6.84	1082473[b]	514			

Numbers in the first column correspond to the ones labeled in Figs. 2 and 3. Identified peaks matched both spectrum and retention time of standards. Unidentified peaks are tentative matches to the NIST library.

[a] Areas were calculated from the most intense (quan) ion in the spectrum of each respective compound.

[b] Areas were calculated from reconstituted ion chromatogram (RIC).

2.5. Data analysis

Every breath sample was analyzed within 8 h from the time of collection. During that time samples were refrigerated at 4 °C. A user library was created for breath compounds with very short (0.5 s) extractions from the headspace of pure solvents. Peaks were considered “identified” when their mass spectral fit values were at the default value of 700 or above and their respective retention time matched the retention times (±4 s) of the compounds in the user library. Peaks under the category of “unidentified” represent the best match to the US National Institute for Standards

and Technology (NIST) spectral data base library. Once the peaks on all the chromatograms were identified, the highest area count value for compounds in the blank samples were used to compare with those in the breath samples for the corresponding day and particular sampling device. To error on the side of caution, compounds found in ambient air blanks were also compared to the breath samples if their values were higher than the device blanks or trip blanks for a particular sampling day. All area counts were calculated from the single ion chromatogram of the most intense ion (quan ion) from the spectrum of each chemical. It was possible to separate areas for coeluting compounds by using the quan ion. Compounds were reported as breath components only if their area counts were three times greater than the highest blank value. Typical chromatograms of a breath sample and its corresponding blank samples are shown in Fig. 2 with listed breath and background compounds in Table 2. An interference (compound 9) tentatively identified as tetramethyl silane is a label for two peaks in a some of the chromatograms. It is probable that one peak is tetramethyl silane and the other is another silane with only slightly different structure.

3. Results

A total of 21 VOCs were detected in cattle breath. Heptane, octanal, acetaldehyde, 2,3-butadione, isovaleric acid, decanal, hexanoic acid, phenol, toluene, propionic acid, acetic acid, acetophenone, hexane, isopropyl alcohol, nonane, octane, dodecane, acetone, styrene, tetradecane, and methyl ethyl ketone were detected in cattle breath. Five compounds, acetone, tetradecane, acetic acid, methyl ethyl ketone, and decanal, have been previously detected in cattle breath [17–19]. An extraction time of 15 min resulted in adequate detector responses for the detection of the 21 compounds. A typical chromatogram contains peaks for some but not all of the 21 breath compounds (Fig. 3). The frequency of detection of these compounds is displayed in Table 3. Octane was the only breath compound detected at all the 7 days of sampling, but was present in only 26% of the triplicate samples. Acetone was present in almost 50% of all the SPME samples taken and was detected with at least one fiber in six of the seven sampling events. Octane, toluene, acetone, methyl ethyl ketone, and 2,3-butadione were individually present in at least 25% of all the SPME samples taken. There were five compounds, isopropanol, hexane, nonane, acetophenone, and acetic acid, which were found on only 1 day of sampling with one fiber. Cattle eructate gases from the rumen and expel them in expired breath to relieve pressure in the rumen [22]. Six compounds detected in cattle breath (toluene, octanal, acetic acid, propionic acid, isovaleric acid, and hexanoic acid), were also found in the headspace of ruminal culture [23] using SPME and possibly originated from the rumen. Interferences found in ambient, trip, and device blanks usually consisted of silanes, polyaromatic hydrocarbons, and others that probably came from the preservation setup.

Chi-square comparison of the frequencies of detection for all 21 compounds was performed (Table 4). Statistical analyses using Chi-square test on the frequency of detection of each VOC in each group was performed because the MS responses were not considered to be necessarily related to the VOC concentrations. Eight of the 21 compounds were shown to be significantly associated with one of the two treatment classes, i.e. sick or healthy cattle. The presence of acetaldehyde ($P \leq 0.05$) and decanal ($P \leq 0.10$) were associated uniquely with the clinically morbid steers while heptane, octanal, 2,3-butadione, hexanoic acid, and phenol were associated with the healthy steers at $P \leq 0.10$. The complexity of cattle breath VOC mixtures at low concentrations makes collection and analysis a challenging task. It may be possible to discern sick and healthy animals with greater ease with the aid of an electronic nose or some other

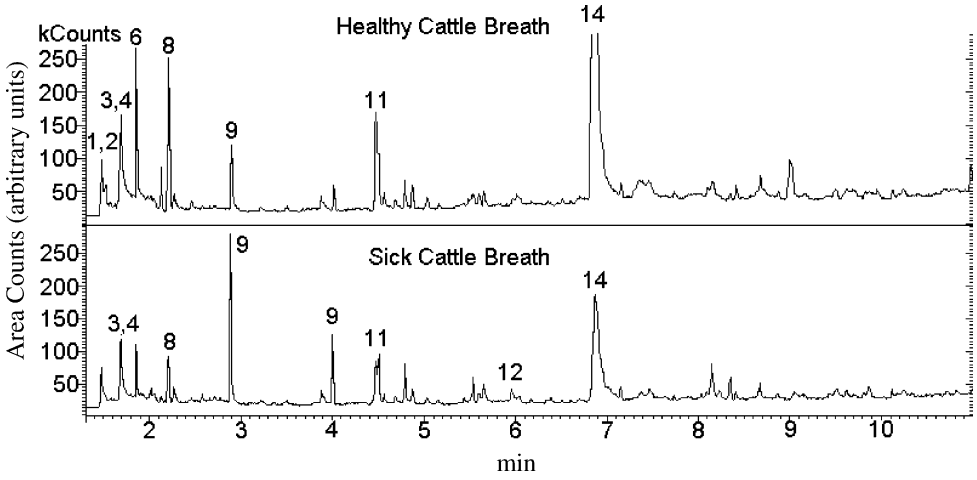

Fig. 3. Comparison of chromatograms of breath samples from healthy and sick steers. Peaks 1 and 2 are mixtures of hexane and heptane. Peaks 3 and 4 are simultaneously eluted acetone and acetaldehyde. Peak numbers correspond to those in Table 2.

Table 3
Summary of VOCs identified in bovine breath

	Day 1	Day 3	Day 5	Day 11	Day 15	Day 17	Day 19	Total detection	Percentage distribution	No. of days found in 7 days of sampling
Alcohols										
Isopropanol					1			1	0.79	1
n-Alkanes										
Hexane	1							1	0.79	1
Heptane	2	3	1		1	2	3	12	9.52	6
Octane	3	9	7	4	6	2	2	33	26.19	7
Nonane				1				1	0.79	1
Dodecane							6	6	4.76	1
Tetradecane	4		1					5	3.97	2
Aromatic compounds										
Toluene	2	1		5	9	14	13	44	34.92	6
Phenol	1						2	3	2.38	2
Styrene	4				1			5	3.97	2
Ketones										
Acetone	17	18	5		1	13	6	60	47.62	6
Methyl ethyl ketone	10	7	10		2	9	6	44	34.92	6
2,3-butadione	2	4			5	12	11	34	26.98	5
Acetophenone	1							1	0.79	1
VFAs										
Acetic acid							1	1	0.79	1
Propionic acid	6	1	5				1	13	10.32	4
Isovaleric acid	2	5						7	5.56	2
Hexanoic acid				3				3	2.38	1
Aldehydes										
Acetaldehyde	5	1	4	2	1	6	2	21	16.67	7
Octanal						5		5	3.97	1
Decanal		1			1		1	3	2.38	3

Frequency of VOC detection, percentage distribution, and sampling day detection are shown for compounds categorized by functional groups.

Table 4
Chi-square values and probabilities (*P*)

Compound	Chi-square value (*Z*)	*P*-values	Healthy total (all days)	Sick total (all days)
Heptane	5.99	0.03	10	2
Octanal	5.41	0.03	5	0
Acetaldehyde	4.69	0.05	6	15
2,3-Butadione	4.07	0.05	22	12
Isovaleric acid	3.93	0.05	6	1
Decanal	3.41	0.1	0	3
Hexanoic acid	3.41	0.1	3	0
Phenol	3.41	0.1	3	0
Toluene	2.27	0.25	18	26
Propionic acid	2.23	0.25	4	9
Acetic acid	2.02	0.25	0	1
Acetophenone	2.02	0.25	0	1
Hexane	2.02	0.25	1	0
Isopropanol	2.02	0.25	1	0
Nonane	2.02	0.25	0	1
Octane	1.07	0.25	19	14
Dodecane	0.88	0.5	2	4
Acetone	0.54	0.5	28	32
Styrene	0.42	0.75	2	3
Tetradecane	0.42	0.75	2	3
Methyl ethyl ketone	0.17	0.9	23	21

Values of *P* indicated the probability that the detected compounds were not dependent on the health of the animals.

hand held device that is set up to handle complicated mixtures of some of the 21 compounds listed in this research. Field portable GC systems could be taken on-site, and results could be made available with almost real time sampling to make the method more practical for receiving, testing, and treating cattle as they arrive at the feedlot.

4. Conclusions

A simple sampling system, combined with SPME-based sampling and analysis with GC–MS was useful for the detection of volatile organic compounds in bovine breath gases. The device for on-site, noninvasive animal breath sample collection was designed, built, and tested, and is lightweight, versatile and applicable to a variety of domestic animals. This method is also a relatively noninvasive method that allowed the animals to breathe comfortably. The DVB–Carboxen–PDMS 50/30 μm coating, PTFE caps, and refrigeration provided limited repeatability. A sampling time longer than 15 min might be necessary for the collection of large enough masses to reliably reproduce results. Transportation of fibers and their subsequent storage may have been a source of analyte losses. After improvements in the storage of fibers are completed, SPME-based sampling of animal breath has a potential to become a noninvasive on-site diagnostic and research tool. Several VOCs appear to be good candidates for biomarkers of stress due to respiratory disease. The results suggest that noninvasive heath screening using SPME-based breath analyses could be a useful diagnostic tool for animals and humans.

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